Biochemical Pharmacology, Vol. 24, pp. 1771–1776. Pergamon Press, 1975. Printed in Great Britain.

EFFECT OF WASHING THE HEPATIC MICROSOMAL FRACTION IN SUCROSE SOLUTIONS AND IN SUCROSE SOLUTION CONTAINING EDTA UPON THE METABOLISM OF FOREIGN COMPOUNDS

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(*Received* 9 *January* 1975; *accepted* 18 *March* 1975)

Abstract—Washing the hepatic microsomal fraction of the rat in 0·25 M sucrose containing 0·05 M Tris buffer (pH 7·4) resulted in a decrease in the metabolism and binding to cytochrome P-450 of Type I substrates, although not of a Type II substrate. The levels of microsomal cytochrome b_5, cytochrome P-450 and NADPH-cytochrome *c* reductase were unchanged, whilst the activity of NADPH-cytochrome P-450 reductase was reduced. The inhibitors of lipid peroxidation EDTA and Mn^{2+} added to the washing medium prevented the decrease in the metabolism of Type I substrates. It is suggested that the effects of washing may be related to the increase in the level of peroxidised microsomal lipid which could lead to a selective destruction of the Type I binding site. EDTA added to the washing medium also produced an increase above control values, in the metabolism and binding of both Type I and Type II substrates, which may be related to the apparent increase in the amount of microsomal cytochrome P-450. Washing the microsomal fraction almost completely abolished the ability of acetone to enhance aniline hydroxylation. It is concluded that the effects of acetone are not due to the displacement of endogenous substrates bound to the microsomal fraction.

The hepatic microsomal fraction used in the study of the metabolism of foreign compounds by the mixed function oxidase may be freed from adhering non-microsomal protein by washing in 0·15 M KCl. This is a procedure adopted routinely by some workers with no apparent detrimental effects[1–6]. It was, however, found in this laboratory that washing the microsomal fraction in 0·25 M sucrose containing 0·05 M Tris buffer (pH 7·4), a medium also used routinely in the preparation of the microsomal fraction [7–9], resulted in a decreased ability of the mixed function oxidase to metabolise certain substrates [10]. These observations have been extended in the present study.

MATERIALS AND METHODS

Preparation and washing the microsomal fraction. The microsomal fraction was prepared from livers of 200-g fed female Wistar rats by the method of Ernster *et al.* [11] in 0·25 M sucrose containing 0·05 M Tris buffer (pH 7·4) (buffered sucrose medium), resuspended with a loose hand-operated pestle and adjusted to give a protein concentration of 10 mg/ml. Protein was measured by the method of Lowry *et al.* [12] using crystalline bovine serum albumin as a standard. When required an aliquot of the microsomal fraction was washed by diluting with 3 vol of ice-cold sucrose buffer, stood at 4° for 30 min and the microsomes reharvested by centrifuging at 105,000 *g* for 60 min at 4°. The microsomal pellet was suspended in sucrose buffer at a protein concentration of 10 mg/ml as before, and its properties compared with those of the remaining unwashed fraction, which had been stored in buffered sucrose medium at 4° and which acted as a control.

Assays. The metabolism of foreign compounds by the microsomal fraction was measured over 30 min at 37° with a reaction mixture containing 4 mg microsomal protein, Tris buffer (pH 7·4) 150 μmoles, $MgCl_2$ 15 μmoles, nicotinamide 50 μmoles, DL-isocitrate 20 μmoles, isocitrate: $NADP^+$ oxidoreductase 2U, $NADP^+$ 1 μmole and either aminopyrine (4-dimethylaminoantipyrine) 10 μmoles, or aniline 5 μmoles, all in a final incubation vol of 2 ml. Alternatively, NADPH was added directly to the reaction mixture as 3 × 2-μmole aliquots at 10-min intervals as described by Mazel [7], instead of employing the NADPH generating system. Formaldehyde formed from aminopyrine was trapped with semicarbazide 10 μmoles, and measured by the method of Nash [13]. *p*-Aminophenol formed from aniline was measured by the method of Schenkman *et al.* [14]. The cytochrome b_5 and cytochrome P-450 content of the microsomal fraction was determined by the method of Dallner [15]. The molar extinction coefficient of cytochrome b_5 was taken as 171 cm^{-1} mM^{-1} and cytochrome P-450 as 91 cm^{-1} mM^{-1} [7]. NADPH-cytochrome *c* reductase was measured by the method of Mazel [7] and NADPH-cytochrome P-450 reductase by the method of Gigon *et al.* [16]. The rate of cytochrome P-450 reduction was measured over the first 15 sec and expressed as nmoles cytochrome P-450 reduced/mg microsomal protein per min. The spectral changes produced by the interaction of

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foreign compounds with microsomal cytochrome P-450 were measured by the method of Schenkman *et al.* [14] using 80 μM SKF 525-A* (producing a Type I spectral change), 5 mM aniline (producing a Type II spectral change) and 0·7 M acetone (producing a reverse Type I spectral change). Microsomal lipid peroxidation was measured as the formation of malonaldehyde by the method of Bidlack *et al.* [17], both as peroxides present in the microsomal fraction and as peroxides formed during incubation of the microsomal fraction with the NADPH generating system at 37 for 30 min, in the absence of other substrates, or in the presence of 5 mM aminopyrine or 2·5 mM aniline.

Statistical treatment of results. Groups of data were subjected to a *t*-test [18] to determine if a significant difference existed at the 5% level between the means of groups of data. The values given are means ± S.E.

Materials. SKF 525-A was supplied by Smith Kline & French Laboratories Ltd., Welwyn Garden City, Herts., U.K. NADP$^+$, NADPH and cytochrome *c* were purchased from the Boehringer Corp. (London) Ltd., Ealing, London W5 2TZ, U.K., aminopyrine from Ralph N. Emanuel Ltd., Wembley, Middx., U.K., isocitrate: NADP+ oxidoreductase, DL-isocitrate trisodium salt and crystalline bovine serum albumin from the Sigma Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K., and sucrose, acetone, aniline HCl and EDTA disodium from B.D.H. Chemicals Ltd., Poole, Dorset, U.K., all as the purest grades available.

RESULTS

Microsomal metabolism of foreign compounds. The effect of washing the hepatic microsomal subcellular fraction in 0·25 M sucrose containing 0·05 M Tris buffer (pH 7·4) upon its ability to metabolise aminopyrine and aniline is shown in Table 1. Washing resulted in a 28 per cent decrease in aminopyrine demethylation but had no effect upon aniline hydroxylation. EDTA 10^{-3} M, added to the washing medium produced a 52 per cent increase in aminopyrine demethylation and a 58 per cent increase in aniline hydroxylation above control values. A similar pattern of changes was observed when the microsomal fraction was washed in 0·25 M sucrose, without any Tris buffer. NADPH added directly to the reaction mixture also revealed the same pattern of changes, thus the effects observed could not be accounted for in terms of a change in the activity of the NADPH generating system. The microsomal fraction prepared from male rats showed the same changes as the microsomal fraction from female rats.

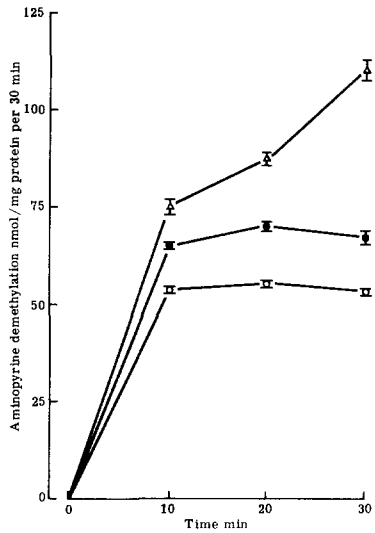

Fig. 1. Effect of washing the microsomal fraction upon the time course of aminopyrine demethylation. Aminopyrine demethylation was measured as described in the text with the pooled microsomal fraction from four rats. Control (●—●), washed with buffered sucrose medium (○ ○) and washed with buffered sucrose medium containing 10^{-3} M EDTA (△ △). Values are the means of six determinations and bars represent S.E.

The effect of washing the microsomal fraction upon aminopyrine demethylation after different times of incubation is shown in Fig. 1. Washing in the buffered sucrose medium had little effect upon the stability of the mixed function oxidase during the incubation. The rate of aminopyrine demethylation was depressed uniformly over the 30 min of incubation. The addition of 10^{-3} M EDTA to the washing medium resulted in a smaller fall in the rate of aminopyrine demethylation during the later stages of the incubation. EDTA thus appears to increase the stability of the mixed function oxidase during incubation.

Components of the microsomal mixed function oxidase. The effect of washing the microsomal fraction upon the components of the mixed function oxidase is shown in Table 2. Washing in the buffered sucrose

* 2-diethylaminoethyl 2,2-diphenylvalerate HCl.

Table 1. The effect of washing the microsomal fraction in buffered sucrose medium or in buffered sucrose medium containing EDTA upon the metabolism of aminopyrine and aniline

	Aminopyrine demethylation (nmoles/mg protein per 30 min)	Aniline hydroxylation (nmoles/mg protein per 30 min)
Control	67·3 ± 7·3	6·6 ± 0·7
Washed microsomes		
Sucrose	48·5 ± 2·0*	6·8 ± 0·3
EDTA 10^{-3} M	102·3 ± 5·4†	11·0 ± 1·0†

* $P < 0.05$.

† $P < 0.01$.

The hepatic microsomal fraction was washed, as described in the text, in 0·25 M sucrose containing 0·05 M Tris buffer (pH 7·4), with and without 10^{-3} M EDTA. Values are mean ± S.E. of six experiments.

Table 2. The effect of washing in buffered sucrose medium or in buffered sucrose medium containing EDTA upon the components of the microsomal mixed function oxidase

	Protein (mg/g liver)	Cytochrome b_5 (nmoles/mg protein)	Cytochrome P-450 (nmoles/mg protein)	NADPH-cytochrome *c* reductase (nmoles cyt. *c* reduced/mg prot. per min)	NADPH-cytochrome P-450 reductase (nmoles cyt. P-450 reduced/mg prot. per min)
n	20	4	4	4	4
Control	7·62 ± 0·25	0·462 ± 0·030	0·799 ± 0·020	62·6 ± 4·5	1·345 ± 0·110
Washed microsomes					
Sucrose	6·00 ± 0·20†	0·408 ± 0·035	0·744 ± 0·039	68·0 ± 3·7	0·365 ± 0·085†
EDTA 10^{-3} M	5·27 ± 0·10†	0·551 ± 0·028*	1·144 ± 0·022†	72·1 ± 5·2	1·358 ± 0·091

* $P < 0.05$.
† $P < 0.01$.
The hepatic microsomal fraction was washed as described in the text, in 0·25 M sucrose containing 0·05 M Tris buffer (pH 7·4), with and without 10^{-3} M EDTA. Values are mean ± S.E.

medium had no effect upon the levels of cytochrome b_5, cytochrome P-450 or NADPH-cytochrome *c* reductase, when expressed per mg microsomal protein. It is thus probable that the loss of protein upon washing in sucrose buffer represents a non-specific loss from all the microsomal protein fractions. There was, however, a 73 per cent decrease in the initial rate of the reduction of cytochrome P-450 by NADPH, although the second slower phase of the reduction did not appear to be affected (Fig. 2). The addition of 10^{-3} M EDTA to the washing medium prevented the decrease in the initial rate of the reduction of cytochrome P-450 by NADPH and even protracted this initial phase of the reduction. There was also a 43 per cent increase in the levels of microsomal cytochrome P-450 which is more than could be accounted for even assuming a specific loss of microsomal protein.

Spectral changes. The spectral change produced by SKF 525-A, a Type I compound [14], was reduced 27 per cent by washing the microsomal fraction in the buffered sucrose medium whilst there was no effect on the spectral change produced by aniline, a Type II compound [19] (Table 3). The addition of 10^{-3} M EDTA to the washing medium resulted in a 40 per cent increase in the Type I spectral change and a 22 per cent increase in the Type II spectral change, compared to the unwashed microsomal fraction. The reverse Type I spectral change produced by acetone [20], which it has been suggested may represent the displacement of endogenous substrates bound to the Type I site [4], was completely absent after washing the microsomal fraction in sucrose buffer, whilst the addition of 10^{-3} M EDTA to the washing medium partly prevented the decreased reverse Type I spectral change.

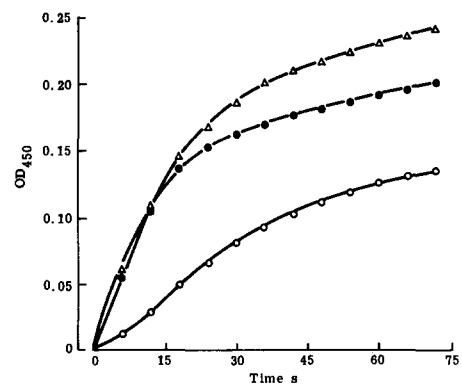

Fig. 2. NADPH-cytochrome P-450 reductase in the washed hepatic microsomal fraction. The formation of the reduced cytochrome P-450–CO complex was measured at 450 nm with the pooled microsomal fraction from four rats. Control (●—●) washed with buffered sucrose medium (○—○) and washed with buffered sucrose medium containing 10^{-3} M (△—△). The microsomal protein concentration was 5 mg/ml. Each point is the mean of four determinations.

Table 3. The effect of washing in buffered sucrose medium or in buffered sucrose medium containing EDTA upon the spectral changes in hepatic microsomes

	Type I ($E_{385-416}$/g)	Type II ($E_{429-395}$/g)	Reverse Type I ($E_{418-390}$/g)
n	6	6	6
Control	25·0 ± 0·5	20·0 ± 0·8	7·6 ± 0·5
Washed microsomes			
Sucrose	18·3 ± 0·5†	19·6 ± 0·4	0·1 ± 0·0†
EDTA 10^{-3} M	35·0 ± 1·8†	24·2 ± 1·9*	5·1 ± 0·6*

* $P < 0.05$.
† $P < 0.01$.
The hepatic microsomal fraction was washed as described in the text, in 0·25 M sucrose containing 0·05 M Tris buffer (pH 7·4), with and without 10^{-3} M EDTA. The values represent the mean ± S.E. of the peak to trough spectral change caused by addition of SKF 525-A 80 μM (Type I), aniline 5 mM (Type II) or acetone 0·7 M (Reverse Type I) to a cuvette containing microsomes 2 mg/ml. For convenience the results are expressed per g microsomal protein.

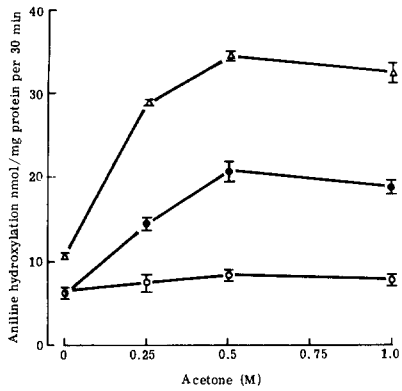

Fig. 3. Acetone enhancement of aniline metabolism by the washed hepatic microsomal fraction. Aniline hydroxylation was measured as described in the text with the pooled microsomal fraction from four rats. Control (●—●), washed with buffered sucrose medium (○—○) and washed with buffered sucrose medium containing 10^{-3} M EDTA (△– △). Acetone was added directly to the reaction mixture. Values are the means of four determinations and bars represent S.E.

Acetone enhancement of aniline hydroxylation. Washing the microsomal fraction in the buffered sucrose medium almost completely abolished the ability of acetone added directly to the reaction mixture to enhance aniline hydroxylation (Fig. 3). The addition of 10^{-3} M EDTA to the washing medium prevented the loss of acetone enhancement. The possibility that acetone was exerting its effects upon aniline hydroxylation by displacing endogenous substrates bound to cytochrome P-450 was examined by washing the microsomal fraction in sucrose buffer containing 0·5 M acetone which should have removed at least some of the endogenous substrates. The microsomal fraction then lost its ability to respond to acetone added to the reaction mixture. If 10^{-3} M EDTA was also added to the washing medium the microsomal fraction retained its full susceptibility to acetone enhancement. It is thus unlikely that acetone was producing enhancement by displacing endogenous substrates, unless EDTA was able to prevent this displacement during the washing process. Acetone was, however, able to enhance aniline hydroxylation with 10^{-3} M EDTA present in the reaction mixture, provided that NADPH was added directly and not provided by the generating system, which is inhibited by EDTA. This would not have been possible if EDTA was able to prevent the displacement of endogenous substrates. Washing the microsomal fraction in 0·15 M KCl containing 0·5 M acetone also failed to prevent the enhancement of aniline hydroxylation by acetone added directly to the reaction mixture.

Microsomal lipid peroxidation. EDTA is an inhibitor of lipid peroxidation [21] and it was found that 10^{-3} M $MnCl_2$, also an inhibitor of lipid peroxidation [22] when added to the washing medium protected against the decrease in aminopyrine demethylation. Unlike EDTA, however, $MnCl_2$ failed to increase aminopyrine demethylation or aniline hydroxylation above control values. Washing the microsomal fraction in a buffered sucrose medium containing 10^{-3} M $FeCl_2$, which has been reported to stimulate lipid peroxidation [23], resulted in a decrease in aminopyrine demethylation to 45·3 ± 2·0 nmoles/mg per 30 min ($n = 6$), which was not greater than caused by washing alone ($P > 0.05$). Aniline hydroxylation was, however, still unaffected.

The effect of washing the microsomal fraction upon the peroxidation of microsomal lipid which occurs spontaneously at 4° and upon NADPH dependent lipid peroxidation which occurs during incubation at 37° is shown in Table 4. Washing the microsomal fraction in the buffered sucrose medium increased both the spontaneous and the NADPH dependent lipid peroxidation. The addition of 10^{-3} M EDTA to the washing medium prevented the increase in NADPH dependent lipid peroxidation but did not completely block the increase in spontaneous lipid peroxidation. Similar changes in NADPH dependent lipid peroxidation were observed in the presence of aniline and aminopyrine, despite the fact that both substrates produced a small, overall inhibition in lipid peroxidation.

Washing with post microsomal supernatant. Washing the microsomal fraction in the post microsomal supernatant produced no decrease in aminopyrine demethylation despite the fact that the post microsomal supernatant was prepared in the buffered sucrose medium. The levels of aminopyrine demethylation

Table 4. The effect of washing the microsomal fraction in buffered sucrose medium or in buffered sucrose medium containing EDTA upon microsomal lipid peroxidation

	Spontaneous lipid peroxidation (E_{532}/mg protein)	NADPH-dependent lipid peroxidation (E_{532} mg protein)
Control	0·072 ± 0·003	0·143 ± 0·005
Washed microsomes		
Sucrose	0·124 ± 0·006†	0·182 ± 0·010†
EDTA 10^{-3} M	0·088 ± 0·008*	0·143 ± 0·010

* $P < 0.05$.
† $P < 0.01$.
The hepatic microsomal fraction was washed as described in the text, in 0·25 M sucrose containing 0·05 M Tris buffer (pH 7·4), with and without 10^{-3} M EDTA. Lipid peroxidation was measured both as peroxides present in the microsomal fraction and as peroxides formed during incubation of the microsomal fraction with a NADPH generating system at 37° for 30 min, in the absence of other substrates. Values are mean ± S.E. of six experiments.

were even increased above control values to 77·0 ± 1·1 nmoles/mg per 30 min (n = 4, $P < 0.01$) whilst aniline hydroxylation was unchanged at 5·8 ± 0·3 nmoles/mg per 30 min (n = 4, $P > 0.05$). Heating the post microsomal supernatant at 100° for 15 min, abolished the stimulation of aminopyrine demethylation above control levels, giving values of 67·1 ± 1·2 nmoles/mg per 30 min (n = 4, $P > 0.05$).

Washing with 0·15 M KCl. None of the changes in metabolising activity so far reported were observed when the microsomal fraction was washed with 0·15 M KCl. This agrees with the observation of Cinti *et al.* [4] that washing the microsomal fraction in 0·15 M KCl had no effect upon the Type I or reverse Type I spectral changes.

DISCUSSION

Washing the hepatic microsomal fraction in 0·25 M sucrose containing 0·05 Tris buffer (pH 7·4) resulted in a 28 per cent reduction in the metabolism of aminopyrine, a Type I substrate [19], whilst the metabolism of aniline, a Type II substrate [19], was unchanged. The levels of microsomal cytochrome b_5, cytochrome P-450 and NADPH-cytochrome *c* reductase were unaffected whilst NADPH-cytochrome P-450 reductase was decreased over 70 per cent by washing in sucrose buffer. The reduction of cytochrome P-450 has been suggested to be the rate limiting step in the microsomal metabolism of foreign compounds [16, 24, 25], and this could then account for the decrease in the metabolism of aminopyrine, although it is difficult to see why, if this were the only mechanism involved, the metabolism of aniline was not also reduced. Washing in the buffered sucrose medium also decreased the Type I spectral change produced by the binding of substrates to cytochrome P-450 whilst the Type II spectral change was unaffected. The binding of substrates to the Type I site facilitates the flow of electrons within the haemoprotein complex [26] and destruction of the Type I binding site results in a decreased metabolism of Type I substrates [26–28]. It is thus possible to explain the decrease in the metabolism of aminopyrine after washing the microsomal fraction in the buffered sucrose medium in terms of a decrease in the binding of substrates to the Type I site. It is not known, however, if there is a direct relationship between the decreased Type I binding and the decrease in NADPH-cytochrome P-450 reductase. Type I substrates facilitate the reduction of cytochrome P-450 by NADPH [16] and a removal of endogenous substrates bound to the Type I site by washing in sucrose buffer might thus lead to the observed decrease in the rate of cytochrome P-450 reduction. It was found that washing the microsomal fraction in the buffered sucrose medium resulted in a complete abolition of the reverse Type I spectral change, which it has been suggested may represent a displacement of endogenous substrates from the Type I site.

Inhibitors of lipid peroxidation, such as EDTA and Mn^{2+}, added to the washing medium prevented the decrease in aminopyrine demethylation, whilst Fe^{2+}, which stimulates lipid peroxidation, aggravated the decrease in aminopyrine demethylation. It was found that washing in the buffered sucrose medium resulted in an increase in NADPH dependent peroxidation of microsomal lipid during incubation of the microsomal fraction at 37° for 30 min, and also an increase in the level of peroxidised lipid in the freshly prepared microsomal fraction. Both processes could account for a decrease in the metabolism of foreign compounds by the microsomal fraction. Direct competition for NADPH between the pathways of lipid peroxidation and foreign compound metabolism has been reported [22, 29, 30], and the addition of inhibitors of lipid peroxidation to the reaction mixture will stimulate the metabolism of foreign compounds [31]. The metabolism of both Type I and II substrates is, however, stimulated under these conditions and it is thus unlikely that in the present study an increase in NADPH dependent lipid peroxidation could account for the selective decrease in the metabolism of Type I substrates. The other possibility is that the spontaneous peroxidation of microsomal lipids, possibly phosphatidylcholine, during the washing with sucrose buffer could produce the observed changes. Phosphatidylcholine is an essential component of the Type I binding site and removal of phosphatidylcholine by incubation of the microsomal fraction with phospholipase C [26] or by isooctane extraction [28] leads to a selective decrease in the binding of substrates to the Type I site and in the metabolism of Type I substrates. It is of course possible that the destruction of phosphatidylcholine during incubation at 37° could account for the decreased aminopyrine metabolism, although a decreased Type I spectral change was observed in the microsomal fraction prior to incubation at 37°.

EDTA added to the washing medium almost completely prevented the increased lipid peroxidation, whilst the levels of both aminopyrine demethylation and aniline hydroxylation were increased above the control levels. The additional stimulatory effect of EDTA may be related to the increase in the levels of microsomal cytochrome P-450 and the increased binding of both Type I and II substrates. The increase in cytochrome P-450 was greater than could be accounted for simply in terms of a specific loss of other microsomal proteins, although EDTA is known to detach ribosomes and other proteins from the microsomal membranes [32]. The increase in the amount of cytochrome P-450 need not in itself lead to an increase in the metabolism of foreign compounds since it is thought that the rate limiting step in the metabolism of foreign compounds is the reduction of cytochrome P-450 by NADPH [16, 24, 25]. EDTA in the washing medium led to a protracted initial rapid phase in the reduction of cytochrome P-450 by NADPH. It may be, therefore, that the effects of EDTA in increasing the metabolism of both Type I and II substrates above control values, can be explained by an increase in the pool of readily reducible cytochrome P-450. It is also possible that EDTA carried over in the microsomal pellet from the washing medium could act to inhibit NADPH dependent lipid peroxidation during incubation at 37° and thus lead to the observed increase in the metabolism of aminopyrine and aniline. There was no direct evidence, however, that lipid peroxidation was lower in the microsomal fraction washed in medium containing EDTA than in the control microsomal fraction.

Washing the microsomal fraction in the buffered sucrose medium almost completely abolished the ability of acetone added directly to the reaction mixture to enhance aniline hydroxylation. It is possible that acetone could be producing enhancement by displacing endogenous substrates bound to cytochrome P-450 which act in some way to selectively inhibit the metabolism of Type II substrates. This is, however, unlikely since the effects of acetone were unaltered by washing the microsomal fraction with medium containing acetone, which should have removed endogenous substrates, providing that steps were taken to prevent the destruction of the Type I site during the washing. It is thus probable that the inability of acetone to produce an enhancement of aniline hydroxylation with a microsomal fraction washed in sucrose buffer is related to the decreased binding to the Type I site, the integrity of which is in some way essential for acetone enhancement [33].

The post microsomal supernatant appears to contain a heat stable factor which prevents the decrease in aminopyrine demethylation when the microsomal fraction is washed in sucrose buffer, and also a heat labile factor which stimulates the metabolism of aminopyrine. Both heat stable [34] and heat labile [35] activators of the microsomal metabolism of foreign compounds have been reported to exist in the microsomal supernatant. It is not clear, however, if there is any relationship between these activators and the activators found in the present study.

Acknowledgements—A.R.B. is a Medical Research Council Scholar. We are grateful for a grant from the Wood Boyd Fund of Glasgow University.

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